Modulation of Dopamine D<sub>2</sub> Receptor Signaling by Actin-Binding Protein (ABP-280)

M. LI, J. C. BERMAK, Z. W. WANG, and Q. Y. ZHOU
Department of Pharmacology, University of California, Irvine, California
Received June 28, 1999; accepted November 18, 1999

**ABSTRACT**

Proteins that bind to G protein-coupled receptors have recently been identified as regulators of receptor anchoring and signaling. In this study, actin-binding protein 280 (ABP-280), a widely expressed cytoskeleton-associated protein that plays an important role in regulating cell morphology and motility, was found to associate with the third cytoplasmic loop of dopamine D<sub>2</sub> receptors. The specificity of this interaction was originally identified in a yeast two-hybrid screen and confirmed by protein binding. The functional significance of the D<sub>2</sub> receptor-ABP-280 association was evaluated in human melanoma cells lacking ABP-280. D<sub>2</sub> receptor agonists were less potent in inhibiting forskolin-stimulated cAMP production in these cells. Maximal inhibitory responses of D<sub>2</sub> receptor activation were also reduced. Further yeast two-hybrid experiments showed that ABP-280 association is critically dependent on the carboxy domain of the D<sub>2</sub> receptor third cytoplasmic loop, where there is a potential serine phosphorylation site (S358). Serine 358 was replaced with aspartic acid to mimic the effects of receptor phosphorylation. This mutant (D<sub>2</sub>S358D) displayed compromised binding to ABP-280 and coupling to adenylate cyclase. PKC activation also generated D<sub>2</sub> receptor signaling attenuation, but only in ABP-containing cells, suggesting a PKC regulatory role in D<sub>2</sub>-ABP association. A mechanism for these results may be derived from a role of ABP-280 in the clustering of D<sub>2</sub> receptors, as determined by immunocytochemical analysis in ABP-deficient and replete cells. Our results suggest a new molecular mechanism of modulating D<sub>2</sub> receptor signaling by cytoskeletal protein interaction.

Dopamine D<sub>2</sub> receptors, which belong to the superfamily of G protein-coupled receptors (GPCRs), regulate essential neurobiological and endocrine processes. Hence, the cellular signaling of D<sub>2</sub> receptors has been extensively investigated. Beyond inhibiting adenylate cyclase, D<sub>2</sub> receptors modulate the activities of potassium and calcium channels, phospholipase C, mitogen-activated protein kinase, sodium-proton exchangers, and the release of arachidonic acid (Sibley and Monsma, 1992; Civelli et al., 1993; Neve and Neve, 1997; Missale et al., 1998). These D<sub>2</sub> signaling pathways seem to be regulated in a complex fashion. For instance, protein kinase C (PKC) phosphorylation directs the preferential coupling of D<sub>2</sub> receptors from the inhibition of adenylate cyclase to the release of arachidonic acid (Di Marzo et al., 1993). Cell type specificity is exhibited as D<sub>2</sub> receptors inhibit phospholipase C in pituitary cells, but activate it in fibroblast cells (Vallar et al., 1990). The effector coupling efficiency of D<sub>2</sub> receptors seems also to be regulated by the subcellular localization of these receptors. In particular, presynaptic D<sub>2</sub> autoreceptors found on the axonal terminals of dopaminergic neurons are more sensitive to agonist stimulation than their identical postsynaptic counterparts (Skirboll et al., 1979; Clark and White, 1987; Missale et al., 1998). In light of these differences in both the cell-type specificity and coupling efficiency of D<sub>2</sub> receptors, cellular pathways additional to G protein coupling are likely to be involved in the activity and regulation of D<sub>2</sub> receptor signaling.

Effecter coupling and membrane targeting of GPCRs have been found to be regulated by a variety of protein-protein interactions. A nearly universal mechanism of terminating GPCR signaling is mediated by the binding of arrestins after receptor phosphorylation by GPCR kinases (Krupnick and Benovic, 1998). Recently, proteins that bind to specific members of GPCRs have been identified as unique players in receptor signaling or targeting. For example, the association of β<sub>2</sub> adrenergic receptors with the protein translation initiation factor (eIF-2B) has been shown to enhance the ability of these receptors to activate adenylate cyclase (Klein et al., 1997). β<sub>2</sub> adrenergic receptors also activate sodium-proton exchangers by recruiting regulatory factors in an agonist-dependent but G protein-independent fashion, indicating the

**ABBREVIATIONS:** GPCR, G protein coupled receptor; PKC, protein kinase C; ABP-280, actin-binding protein 280; MBP, maltose-binding fusion protein; GST, glutathione S-transferase; CHO, Chinese hamster ovary; TBS-T, Tris-buffered saline/Tween 20; TEM, Tris/EDTA/MgCl<sub>2</sub>; 6,7-ADTN, (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide; PMA, 4-ß-phorbol-12-myristate-13-acetate; FITC, fluorescein isothiocyanate; PDZ, PSD-95/DlgA/ZO-1.
existence of novel signaling mechanisms distinct from traditional GPCR second messenger pathways (Hall et al., 1998). Moreover, a family of single-transmembrane-domain proteins has been identified as modifying proteins for calciotinin-receptor-like receptors (McLatchie et al., 1998). These single-transmembrane-domain proteins are required for the targeting of calciotinin-receptor-like receptors to the plasma membrane and also determine their ligand specificity. Finally, ATRAP, a novel protein that interacts with the carboxyl-terminal cytoplasmic domain of the angiotensin II type 1 receptor has been found to negatively regulate receptor signaling (Daviet et al., 1999).

We have investigated the possibility that novel protein interactions with the D2 receptor may regulate its signaling or targeting. Here, we show that cytoskeletal protein actin-binding protein 280 (ABP-280) interacts with the third cytoplasmic loop of the D2 receptor. We demonstrate that this association enhances coupling efficiency of D2 receptors to adenylyl cyclase, can be regulated by PKC activation, and seems to play a role in cell surface receptor clustering.

**Materials and Methods**

**Yeast Two-Hybrid.** The third cytoplasmic loop of the human D2 receptor (residues 211-372) was amplified by PCR and subcloned in-frame into the Gal4 DNA-binding domain vector pGBT9 (Clontech, Palo Alto, CA) to generate pGBT9-D2. pGBT9-D2 was used to screen a human brain cDNA library constructed in the Gal4 activation domain vector pACT2 (Clontech). Library plasmid DNAs were isolated by plating 5 × 106 independent clones on 80 150-mm LBA plates. Handling and transformation of yeast (strain Y190) were performed as described (Matchmaker Two-hybrid System protocol; Clontech). Briefly, yeast cells were sequentially transformed with pGBT9-D2 and 300 µg of library plasmid DNA using the lithium acetate method. The final transformation mixture (2.6 × 109 yeast plasmid transformants) was plated onto 100 150-mm synthetic dextrose agar plates lacking tryptophan, leucine, and histidine but containing 20 mM 3-amino-triazole and allowed to grow for a week at 30°C. Robust colonies were restreaked on fresh plates and tested for binding activity with strain Y190. Wells that were incubated with immobilized MBP-ABP280 at 4°C overnight in ice-cold M9 at a setting of 5 for 10 sec on ice. Radioligand binding was performed in a volume of 1 μl using approximately 200 µg of total protein per tube. Competition binding assays were performed using 0.1 to 0.3 nM [3H]spiperone (99 Ci/mmol; Amersham) and various concentrations of competing compounds. Reaction mixtures were then incubated for 1 h at room temperature and terminated by rapid vacuum filtration through GF/B filters presoaked in 0.5% polyethylenimine (Sigma) using a 24-port harvester (Brandel, Montreal, Canada). Filters were washed with 5 ml of TEM buffer, air dried, and individual filter discs were placed in counting vials with 5 ml of scintillation fluid for counting in a Beckman LS-6800 liquid scintillation counter. Data were analyzed using GraphPad software (San Diego, CA).

**Receptor Functional Analyses.** Stably transfected M2 and A2 cells with similar expression levels of D2 receptors were selected. For cAMP assay, cells were seeded 24 h before the assay at a density of 1 × 105 cells/well. Cells were washed with warm HBBS buffer (20 mM HEPES, pH 7.2, 118 mM NaCl, 4.6 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM d-glucose) and incubated for 10 min at 37°C in 2 βM Ro 20–1724 to inhibit cAMP phosphodiesterase. All the cells were stimulated with 10 µM forskolin and increasing concentration of dopamine or (-)-2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydroxanthene hydrobromide (6,7-ADTN; RBI, Natick, MA). For PKC activation experiments, cells were incubated with forskolin (10 µM) and increasing concentrations of 6,7-ADTN in the presence of 100 nM 4-β-phorbol-12-myristate-13-acetate (PMA; Sigma). Drug incubation...
Modulation of Dopamine D2 Receptor Signaling

was carried out at 37°C for 30 min and terminated by the addition of ice-cold 70% ethanol. cAMP samples were collected in Eppendorf tubes. After drying down, cAMP levels in the samples were determined using a sensitive succinylation method. Each cAMP sample was dissolved in 1 ml of ice-cold NaOAc (50 mM, pH 6.2). One hundred microliters of the sample were succinylated by incubating with 15 mM succinic anhydride (dissolved in 25% triethylamine/75% acetone) on ice for 10 min. Succinylation was terminated by the addition of 2 ml of ice-cold NaOAc (50 mM). One hundred microliters of the dilute succinylated sample were incubated with 100 μl anti-cAMP antibody (Sigma) for 4 h at 4°C. An aliquot of 125I-cAMP (0.0045 μCi) (NEN, Boston, MA) was added into each sample. After overnight incubation at 4°C, immunocomplexes were precipitated with 100 μl of 10% bovine serum albumin and 100 μl of 95% ice-cold ethanol. Radioactivity was determined by counting in a Beckman 5500 Gamma counter. This succinylation radiolmmunoassay has a detection limit of 100 fmol cAMP. Data were analyzed using GraphPad software.

### Immunocytochemistry

A7 and M2 melanoma cells grown in 35-mm glass-bottomed culture dishes (MatTek, Ashland, MA) were transiently transfected with amino-terminally FLAG-tagged D2 or D2 receptors. Two days after transfection, cells were washed in PBS and subsequently fixed in 3.7% formaldehyde in PBS for 30 min on ice. After three washes with PBS, cells were blocked in 5% BSA for 1 h. Cells were incubated overnight at 4°C with 1:1000 dilution of rabbit anti-FLAG antibody (Sigma). After three washes, cells were incubated for 1 h at room temperature with Alexa Fluor 488 or Alexa Fluor 568-conjugated secondary antibody (Molecular Probes). Nuclei were counterstained with 1:1000 dilution of DAPI (4',6-diamidino-2-phenylindole) (Sigma) for 5 min. The coverslips were mounted in Mowiol (Calbiochem). Images were acquired with a Zeiss Axioskop 2 MOT microscope with a Zeiss axioCam MRc camera (Carl Zeiss Microscopy, Thornwood, NY) with a 40× or 63× water immersion objective. Images were captured at 14 bit digital resolution using Zeiss Axiosvision 4.3 software. Images were postprocessed using Adobe Photoshop CS5.0 (Adobe Systems, Inc., San Jose, CA). A minimum of 10 fields per condition were analyzed. For each condition, measurements were made from at least 3 different coverslips in at least 2 independent experiments.

### Table 1

<table>
<thead>
<tr>
<th>Protein-protein interactions detected with the yeast two-hybrid assay</th>
<th>HIPS</th>
<th>β-Gal</th>
<th>ONPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACT2-ABP:pGBT9-D2 (211–372)</td>
<td>++</td>
<td>++</td>
<td>65 ± 8.7 (3)</td>
</tr>
<tr>
<td>pGBT9-ABP: pACT2-D2, (211–372)</td>
<td>++</td>
<td>++</td>
<td>93</td>
</tr>
<tr>
<td>pACT2-ABP: pGBT9-D2 (211–343)</td>
<td>++</td>
<td>++</td>
<td>0.06</td>
</tr>
<tr>
<td>pGBT9-ABP: pACT2-D2 (210–375)</td>
<td>++</td>
<td>++</td>
<td>0.1</td>
</tr>
<tr>
<td>pACT2-ABP: pGBT9-D2S358D</td>
<td>++</td>
<td>++</td>
<td>34 ± 6.1 (3)</td>
</tr>
</tbody>
</table>

ONPG, o-nitrophenyl β-D-galactopyranoside. *P < .05, D2S358D versus D2, unpaired Student’s t test.

### Table 2

<table>
<thead>
<tr>
<th>Ligand affinity of dopamine D2 receptor in M2, A7, and CHO cells</th>
<th>K_I (nM)</th>
<th>A7-D2</th>
<th>M2-D2</th>
<th>CHO-D2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>8,900 ± 2,630</td>
<td>10,100 ± 4,970</td>
<td>5,330 ± 2,020</td>
<td></td>
</tr>
<tr>
<td>Dopamine + GTP</td>
<td>25,175</td>
<td>28,455</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>6,7-ADTN</td>
<td>350 ± 70</td>
<td>330 ± 120</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>(−)-Sulpiride</td>
<td>170 ± 40</td>
<td>160 ± 10</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Haloperidol</td>
<td>3.16 ± 0.29</td>
<td>2.41 ± 0.47</td>
<td>1.80 ± 0.60</td>
<td></td>
</tr>
</tbody>
</table>

*GTP concentration was 50 μM; K_I was calculated as the average of two experiments.

ND, not determined.

### Fig. 1

Binding of ABP-280 to GST-D2 in vitro. Bacterial lysates containing GST-D2, GST-D3, and GST-D4 fusion proteins were tested for their abilities to bind to purified MBP-ABP280. Lanes 1 to 4 indicate GST fusion proteins from bacterial lysates (Lysate). Lanes 5 to 8 indicate GST fusion proteins retained after incubation with MBP-ABP280 (Bound).

### Fig. 2

D2 receptor-mediated inhibition of cAMP accumulation in M2, A7, and CHO cells. Dose-response curves for (A) dopamine or (B) 6,7-ADTN inhibition of cAMP accumulation were determined on cells stimulated with 10 μM forskolin and increasing concentrations of dopamine or 6,7-ADTN. Results are expressed as percentage inhibition of forskolin-stimulated cAMP level. Curves are plotted as average ± S.E. from three to five independent experiments. D2 receptor expression levels were 2.48 pmol/mg of protein (M2) versus 2.55 pmol/mg of protein (A7). The stable CHO cell clone used contained receptor levels of 1.97 pmol/mg of protein. No significant change in either agonist or antagonist affinity for D2 receptors was observed in M2, A7, or CHO cells.
1 h and treated with M2 anti-FLAG antibody (10 μg/ml; Eastman Kodak, New Haven, CT) in 5% BSA overnight at 4°C. The plates were then washed three times with PBS and treated with fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Santa Cruz Biotech) for 1 h. The antibody-labeled cells were then rinsed three times for 5 min each before viewing by confocal microscopy. Confocal microscopy was performed on a Bio-Rad MRC confocal laser microscope equipped with a Nikon Diaphot 200 inverted microscope using a Nikon 60×1.40 NA oil-immersion objective. FITC was excited with a 488-nm argon/krypton laser and emitted fluorescence was detected with a 515–540 nm band pass filter.

**Statistical Analysis.** Statistical analysis was carried out by unpaired Student’s *t* test.

**Results**

**Binding of ABP-280 to the Third Cytoplasmic Loop of the Dopamine D2 Receptor.** To identify proteins that bind to the dopamine D2 receptor, a yeast two-hybrid screen was performed using the entire third cytoplasmic loop of the human D2 receptor (long form, residues 211–372). A single clone (represented identically in three distinct colonies) encoding part of ABP-280 (residues 1779–2134, known as ABP repeats 16 to 19) was isolated from a human brain cDNA library. The specificity of the D2-ABP-280 interaction was evaluated by examining the ability of ABP-280 to bind to the entire third cytoplasmic loops of other dopamine receptors. The short form of the D2 receptor bound to ABP-280 to a similar degree as that of the long form (Table 1). Interestingly, the third cytoplasmic loop of the dopamine D2 receptor also interacted strongly with ABP-280. As quantified by the activities of β-galactosidase, ABP-280 bound to the D2 receptor more intensely than the D3 receptor (Table 1). ABP-280 was found not to interact with the third cytoplasmic loops of either the D1 receptor, a third member of the D2 receptor subfamily, or the D1 receptor, which couples to the stimulation rather than inhibition of adenylate cyclase (Table 1).

The specific interaction of D2 and D3 receptors with ABP-280 was further verified by in vitro protein-protein binding. An MBP-ABP280 (residues 1779–2134) fusion protein was purified and incubated with crude GST fusion proteins containing the third cytoplasmic loops of D2, D3, D4, and D1 receptors. Figure 1 shows that GST-D2 and GST-D3 readily bound to MBP-ABP-280, whereas no interaction was observed with GST-D1 and GST-D4 fusion proteins.

**The Association of D2 Receptors with ABP-280 is Essential for the Efficient Coupling of the Receptor to the Inhibition of Adenylate Cyclase.** The availability of human melanoma cell line (M2) that does not express ABP-280 endogenously allows us to assess D2 receptor properties in the absence of ABP-280. As a control, the A7 cell line was generated by stably transfecting M2 cells with ABP-280 cDNA. Clones stably expressing similar levels of D2 receptors in M2 and A7, cells were selected for further receptor binding and activation studies. Radioligand binding experiments revealed similar affinity for several agonists and antagonists in M2, A7, and control D2 transfected CHO cells (Table 2). Fifty-micromolar GTP treatment reduced the dopamine affinity similarly on both M2 and A7 cells, indicating that ABP-280 binding did not seem to affect receptor/G protein interaction directly (Table 2). However, a significant difference was observed in agonist-mediated inhibition of forskolin-stimulated cAMP production. Figure 2 shows that dopamine was less potent in inhibiting forskolin-stimulated adenylate cyclase in M2 than in A7 cells (EC_{50} = 33.8 and 3.1 nM, respectively; Table 3). The maximum inhibition of D2 receptor activation by dopamine was also reduced in M2 cells (42% compared with 78% in A7 cells; Fig. 2, Table 3). Similar results were observed with a synthetic D2 agonist 6,7-ADTN (Fig. 2, Table 3). As a control study, we found that both the potency and maximal inhibition of dopamine in A7 cells were comparable with those found using a CHO cell line (Fig. 2, Table 3) and to published results using other cell lines (Missale et al., 1998). These findings indicate that the D2 receptor is less efficient in coupling to the inhibition of adenylate cyclase in the absence of ABP-280.

**Regulation of the D2 Receptor and ABP-280 Interaction by Protein phosphorylation.** GPIb_{v}, of the GP Ib-IX complex, the platelet von Willebrand factor receptor that mediates the initial attachment of platelets at a site of injury, has been shown to bind to the same region of ABP-280 as does the D2 receptor (Meyer et al., 1998). GPIb_{v} requires a 30-amino acid domain at its carboxyl terminus for ABP association (Andrews and Fox, 1992). This region displays considerable homology with the carboxyl domain of the D2 and D3, but not the D1 or D4, third cytoplasmic loops (Fig. 3). We tested whether this receptor region is critical for binding to ABP-280. Table 1 shows that elimination of this stretch (D2_{314–368}) abolished the ability of the D2 receptor to associate with ABP-280. Within this domain, there exists a conserved serine residue that is a putative PKC phosphorylation site. To determine whether this potential phosphorylation site may be involved in the regulation of the ABP-280-D2 interaction, we replaced this serine residue with aspartic acid (D2_{S355D}) to mimic its phosphorylated state. Table 1 shows that the D2_{S355D} mutant receptor

---

**TABLE 3**

Inhibition of forskolin-stimulated cAMP production of dopamine D2 receptors

<table>
<thead>
<tr>
<th></th>
<th>EC_{50} (nM)</th>
<th>Maximum Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dopamine</td>
<td>6,7-ADTN</td>
</tr>
<tr>
<td>A7-D2</td>
<td>3.09 ± 0.46</td>
<td>0.28 ± 0.06</td>
</tr>
<tr>
<td>M2-D2</td>
<td>33.78 ± 1.08 (4)*</td>
<td>4.90 ± 0.73 (3)*</td>
</tr>
<tr>
<td>CHO-D2</td>
<td>3.17 ± 0.50 (3)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*P < .05, compared with A7-D2 or CHO-D2 cells, unpaired Student’s *t* test.

ND, not determined.
displayed significantly reduced binding to ABP-280 compared with the wild-type receptor.

To elucidate further the functional significance of serine-358, we stably transfected D_2S358D receptors into A_7 and M_2 cells. Figure 4A shows that the agonist potency of D_2S358D was impaired in A_7 cells, with a reduction also in the maximal inhibition of forskolin-stimulated cAMP production (Fig. 4A, Table 4). In M_2 cells, however, no shifts in agonist potency or maximal signaling between wild-type and D_2S358D receptors were observed, indicating that the effects of this mutation are specific only to ABP-containing cells (Fig. 4B; Table 4). Thus, a structural mimic of serine-358 phosphorylation negatively regulates D_2 receptor association with ABP-280 as well as D_2 receptor signaling in a parallel fashion.

Next, we examined the effects of PKC activation on D_2 receptor signaling. Table 4 shows that PMA treatment reduced the agonist potency of D_2 in A_7 but not in M_2 cells. This reduction in potency was similar to that of the D_2S358D phosphorylation mimic observed in A_7 cells. Notably, there was no further shift in agonist potency of the D_2S358D receptor on treatment with PMA (Table 4). Hence, PKC activation can modulate D_2 receptor signaling in a manner consistent with a regulatory role in ABP-280-D_2 association.

**ABP-280 Affects the Cell Surface Expression Pattern of the D_2 Receptor.**

Several intracellular proteins known to

![Fig. 3. Alignment of the peptide sequences from the region of GPIb-α that is known to bind to ABP-280, and the C-terminal region of the third cytoplasmic loops of D_1 to D_4 receptors. All the peptides were compared with GPIb-α peptide. Gray boxes depict conserved amino acid residues. A putative PKC phosphorylation site in D_2 and D_3 receptors was indicated in bold.](image)

![Fig. 4. Dose-response curves of 6,7-ADTN-stimulated inhibition of cAMP accumulation in wild-type (D_2) and mutant (D_2S358D) D_2 receptors. Both receptors were stably transfected into A_7 (A) and M_2 (B) cells. Stable clones with similar receptor expression levels were tested. The expression levels were 1.48 pmol/mg of protein (A_7-D_2) versus 1.6 pmol/mg of protein (A_7-D_2S358D) and 1.45 pmol/mg protein (M_2-D_2) versus 1.3 pmol/mg protein (M_2-D_2S358D). Dose-response curves were determined as in Fig. 2. Data shown are the mean ± S.E. from three independent experiments.](image)

![Fig. 5. Plasma membrane expression of FLAG-D_1 and FLAG-D_2 receptors in transiently transfected A_7 and M_2 cells. D_2 receptor expression levels on A_7 and M_2 cells in this specific experiment are 1.2 pmol/mg protein and 1.0 pmol/mg protein, respectively. FLAG-D_1 receptors displayed membrane clustering in A_7 cells (A), but more uniform surface distribution in M_2 cells (B). In contrast, FLAG-D_2 receptors showed a similar clustering appearance in both A_7 (C) and M_2 (D) cells. Representative viewing fields were chosen from three independent experiments.](image)
interact with cell surface receptors have been found to be involved in receptor targeting (Gomperts, 1996). To visualize the cellular distribution of $D_2$ receptors, we attached a FLAG epitope to the amino-terminus of the receptors (Vickery and von Zastrow, 1999). FLAG-tagged $D_2$ receptors expressed on $A_7$ and $M_2$ cells showed similar affinity to agonist 6,7-ADTN compared with untagged $D_2$ receptors ($K_i = 284 \pm 72$ and $222 \pm 46$ nM, respectively; $n = 3$; Table 2). FLAG-tagged $D_2$ were then transiently transfected into $A_7$ and $M_2$ cells, labeled with FITC-conjugated secondary antibodies and visualized by confocal microscopy. $D_1$ receptors, also tagged with FLAG at the amino terminus, were used as control receptors. Both $D_2$ and $D_1$ receptors displayed clustering in $A_7$ cells (Fig. 5, A and C). However, in ABP-280-deficient $M_2$ cells, although $D_1$ receptors maintained a clustering appearance, $D_2$ receptors were more uniformly distributed along the plasma membrane (Fig. 5, B and D). These results indicate that ABP-280 contributes to $D_2$ receptor clustering on the cell surface and may serve to anchor these receptors in prime locations for efficient cellular response to agonist stimulation.

**Discussion**

In this study, we found that the dopamine $D_2$ receptor binds to ABP-280, a cytoskeleton-associated protein. Several other neuroreceptors have been reported to associate with cytoskeletal elements. For instance, subtypes of the N-methyl-D-aspartate receptor have been shown to bind to $\alpha$-actinin-2, also a member of the actin-binding protein family, in postsynaptic densities of cortical neurons (Wyszynski et al., 1997). In addition, several ligand-gated ion channels are known to be linked to the cytoskeleton via different adaptor proteins (Kirsch and Betz, 1993; Carbonetto and Lindenbaum, 1995; Wang et al., 1999). Most interestingly, recent reports suggest a similar linkage between GPCRs and the cytoskeleton. For example, the carboxyl terminus of the somatostatin receptor has been shown to associate with the cytoskeleton via cortactin-binding protein (Zitzer et al., 1999). Our data indicate that $D_2$ receptors can bind directly to ABP-280 with important functional consequences.

The association between the $D_2$ receptor and ABP-280 was shown to enhance receptor signaling. In $M_2$ melanoma cells that do not express ABP-280, the ability of $D_2$ receptors to inhibit forskolin-stimulated adenylate cyclase activity was greatly reduced. We have further shown that mimicking protein phosphorylation by substitution of serine-358 with aspartic acid within the ABP-association domain reduces the binding ability and signal coupling efficiency of the mutant $D_2$ receptor (Table 1, Table 4). Direct stimulation of PKC with PMA also reduces $D_2$ signaling (Table 4). This phosphorylation regulation thus suggests a dynamic feature of receptor-cytoskeletal interactions and their potential for regulation by physiological stimuli. PKC activation can occur through multiple pathways. In particular, many GPCRs are capable of signaling homologous and heterologous receptor desensitization through PKC-dependent mechanisms (Chuang et al., 1996). However, it is not clear exactly how PKC-mediated phosphorylation mechanisms alter receptor-signaling pathways. Our findings suggest that one of these mechanisms may involve a regulated association with cytoskeletal components.

It is well known that $D_2$ autoreceptors are more sensitive to agonists than their postsynaptic counterparts (Skirboll et al., 1979), but the underlying mechanisms for this differential sensitivity remain elusive. The involvement of distinct $D_2$ receptor protein sequences is unlikely (Skirboll et al., 1979; Clark and White, 1987; Missale et al., 1998). Our results indicate that the differential agonist sensitivity of the presynaptic and postsynaptic $D_2$ receptors may result from differential neuronal compartmental interactions between $D_2$ receptors and ABP-280. It would be interesting to examine whether the in vivo interaction between ABP-280 and the $D_2$ receptor is more prevalent presynaptically than postsynaptically.

The exact mechanism by which ABP-280 modulates the signaling of $D_2$ receptors is still under investigation, although it is likely that this cytoskeletal component acts as a scaffolding protein. By clustering the components of the signaling pathways together, scaffolding molecules can greatly increase the efficiency of receptor-effector coupling. It has been shown that in *Drosophila melanogaster*, INAD, a protein with five distinct PSD-95/DlgAZO-1 (PDZ) domains, serves as a scaffold in the assembly of a highly organized phototransduction pathway that includes receptor, effectors, and regulators, thereby endowing the signaling pathway with extremely high fidelity (Montell, 1998). Our data indicate that ABP-280 assists in the clustering of $D_2$ receptors to specific cell surface locales. Whether these regions are also enriched in signaling intermediates of $D_2$ receptors remains to be determined. Studies have demonstrated that the distribution of G proteins in the plasma membrane is not random (Wang et al., 1989) and that G proteins or adenylylate cyclase could also be attached to components of the cytoskeleton (Graeser and Neubig 1993; Neubig, 1994). Thus by anchoring receptors as well as signaling molecules, the cytoskeleton may ensure rapid and efficient signal transduction.

ABP-280 is an abundant cytoplasmic protein with an amino terminal actin-binding domain of approximately 275 amino acids, followed by 24 tandem repeats, each approximately 96 amino acids in length (Gorlin et al., 1990). In addition, ABP-280 is a pro-